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BACULOVIRUS-BASED EXPRESSION OF AN INSECT VIRAL PROTEIN IN 12 DIFFERENT INSECT CELL LINES

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SUMMARY

The ability of 12 unique lepidopteran insect cell lines from *Anticarsia gemmatalis*, *Heliothis virescens*, *Lymantria dispar* (two lines), *Mamestra brassica*, *Plutella xylostella*, *Spodoptera frugiperda* (two lines), and *Trichoplusia ni* (three lines) to support production of a recombinant polydnavirus (PDV) protein (GiPDV 1.1) expressed using the Bac-to-Bac™ baculovirus expression system was examined. Polydnavirus gene GiPDV 1.1 was cloned into the pFastBac baculovirus vector under the control of the polyhedron promoter, followed by generation of recombinant bacmid–GiPDV 1.1 by site-specific transposition. The ability of each insect cell line to support recombinant PDV gene expression was estimated using reverse transcriptase–polymerase chain reaction and Western blot. Each insect cell line infected with recombinant bacmid–GiPDV 1.1 and tested in this study was capable of supporting and producing recombinant protein. Time course expression analysis showed that 72–96 h after transfection to be the optimal time for harvest of recombinant protein for each insect cell line.

Key words: Glyptapanteles indiensis polydnavirus; baculovirus expression vector; bacmid; insect cell lines; recombinant protein.

INTRODUCTION

Polydnaviruses (PDVs) are segmented double-stranded deoxyribonucleic acid (DNA) viruses found in braconid and ichneumonid endoparasitic wasps. The PDVs are unique among the insect viruses because of their symbiotic relationships with the wasp hosts. Virus replicates only in the calyx region of the wasp ovaries, and virions are stored in the lumen of the oviduct forming calyx fluid (Norton and Vinson, 1983; Theilmann and Summers, 1986). The female wasp injects calyx fluid along with the egg(s) into its lepidopteran host during oviposition. Within the host, viral DNAs infect host cells and viral transcripts are expressed. Viral gene products are involved in the immunosuppressive response of the host to the wasp eggs and are essential for progeny development (Lawrence and Lanzrein, 1993; Stoltz, 1993; Lavine and Beckage, 1995; Strand and Pech, 1995; Beckage, 1998; Webb, 1998; Schmidt et al., 2001). Clearly, it will be important to identify and characterize the viral genes responsible for disruption of host immune response for elucidation of the mechanisms by which physiological systems are targeted by viral genes.

A gene transcript from the braconid *Glyptapanteles indiensis* PDV, designated GiPDV 1.1, is expressed in the hemolymph of gypsy moth, *Lymantria dispar*, soon after parasitization and reaches peak expression 30 min after parasitization (Chen et al., 2003). Gene GiPDV 1.1 was postulated to be an early expressed viral gene involved in early protection of parasitoid egg(s) from host immune systems. Genomic studies showed the GiPDV 1.1 gene hybridizes to four separate GiPDV DNA segments (Chen and Gundersen-Rindal, 2003), indicating multiple gene loci or homologs within the viral genome. This supports the concept that multiple gene loci may increase gene copy number in the absence of PDV replication and may account for increased levels of gene expression (Xu and Stoltz, 1993; Cui and Webb, 1997). Although presumably required for successful parasitism, function of the GiPDV 1.1 gene product within the parasitized host remains to be determined. The recent completion of the full genome sequence of the braconid *Cotesia congregata* polydnavirus (CcBV) (Espagne et al., 2004) revealed genes of the same family as GiPDV 1.1, designated "f1 gene family." These genes that are also of unknown function in CcBV but similar to GiPDV 1.1 had loci on four separate CcBV genome segments.

To facilitate future functional characterization of the GiPDV 1.1 protein and to elucidate the interactions between PDVs and their insect hosts, we have comparatively examined the ability of 12 different insect cell lines to support GiPDV 1.1 gene expression using the Bac-to-Bac baculovirus system (Invitrogen, Carlsbad, CA). The baculovirus expression system has been used widely for the production of agricultural and medically important proteins (Luckow, 1991; Possee, 1997), and insect cell lines have been of particular interest for expression of heterologous genes because they are relatively cheap to maintain and foreign proteins generated in insect cells are correctly processed (Verma et al., 1998; Ikonomou et al., 2003). In contrast to other systems, the Bac-to-Bac system provides efficient generation of recombinant baculoviruses by site-specific transposition inserting a foreign gene into a baculovirus shuttle vec-

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TABLE 1

INSECT CELL CULTURES

Insect species	Cell line designation	Attachment	Tissue source	Medium	Reference
Anticarsia gemmatalis	UFL-Ag-286	None	Embryos	TC-100[a]	Sieburth and Maruniak, 1988
Heliothis virescens	IPLB-HvE6s	None	Embryos	TC-100	Lynn and Shapiro, 1998
H. virescens	IPLB-HvE1a	Very strong	Embryos	TC-100	Lynn and Shapiro, 1998
Lymantria dispar	IPLB-LdEIta	Strong	Embryos	Ex-Cell 400[b]	Lynn et al., 1988
L. dispar	IPLB-LdEp	Strong	Embryos	Ex-Cell 400	Lynn et al., 1988
Mamestra brassica	IZD-MB0503	None	Larval hemocytes	TC-100	Miltenburger et al., 1977
Plutella xylostella	IPLB-PxE2	Strong	Embryos	TC-100	Lynn, pers. comm.
Spodoptera frugiperda	IPLB-Sf21AE	Strong		Ex-Cell 400	Vaughn et al., 1977
S. frugiperda	IPLB-Sf9	Strong	Pupal ovaries	Ex-Cell 400	Summers and Smith, 1987
Trichoplusia ni	TN-368	None	Pupal ovaries	TNM-FH[c]	Hink, 1970
T. ni	IAL-TND1	None	Imaginal discs	IPL-52P[d]	Lynn et al., 1982
T. ni	IPLB-TN-R[b]	Strong	Embryos	TNM-FH	Rochford et al., 1984

[a] Sigma Chemical Co., St. Louis, MO, as modified in Lynn et al. (1988).
[b] JRH Biosciences, Lenexa, KS.
[c] Hink and Strauss (1976); Invitrogen–GIBCO, Carlsbad, CA.
[d] Goodwin and Adams (1980); JRH Biosciences.

tor, a bacmid, rather than generation of recombinant baculovirus in insect cells by homologous recombination (Luckow et al., 1993). The recombinant bacmid propagated in small-scale *Escherichia coli* cultures can be transfected directly into insect cells, with resulting production of proteins that are posttranslationally processed, modified, and targeted to appropriate cellular locations in a manner similar to that of their authentic counterparts (Qian et al., 2004).

Materials and Methods

Insect cell lines. Cell cultures were maintained at 26° C and passaged on a weekly basis as described in Lynn (2002) using the medium and subculture method relevant to the attachment characteristics as listed in Table 1. Cell line identity was confirmed on an annual basis using the Authentikit® isozyme system (Innovative Chemistry, Marshfield, MA).

Construction of recombinant pFastBac–GiPDV 1.1 and production of recombinant bacmid–GiPDV 1.1. A complementary DNA clone containing the GiPDV 1.1 gene (Chen et al., 2003, GenBank accession number: AF414845) was polymerase chain reaction (PCR) amplified to obtain the gene open reading frame and introduce appropriate restriction endonuclease sites. The sequences of forward and reverse primers were 5′-catgccatggcatgc**atg**ttgtgccgc-3′ (*Nco* I site is shown underlined and translational start codon is shown in bold) and 5′-cccaagcttgggtta**tca**aaaaat-3′ (*Hind*III site is shown underlined and translational stop codon is shown in bold), respectively. The PCR was performed using 30 cycles of 94° C for 30 s, 58° C for 30 s, and 72° C for 1 min, followed by a 10 min extension step at 72° C. The PCR products were purified using Wizard PCR Prep DNA Purification System (Promega, Madison, WI) and digested with *Nco*I and *Hind*III. Recombinant pFastBac–GiPDV 1.1 was constructed by inserting GiPDV 1.1 fragment into *Nco*I and *Hind*III sites of donor plasmid, pFastBacHTb (Invitrogen) in which GiPDV 1.1 gene was driven by the polyhedron promoter. The expressed protein from the gene that was cloned into pFastBacHTb vector contained a 6X-histidine amino terminus tag to facilitate protein detection and purification. After ligation, the recombinant plasmid was transformed into competent *E. coli*, DH5α for selection. The nucleotide sequence of the GiPDV1.1 insert was verified.

Recombinant bacmid was produced in the Bac-to-Bac system (Invitrogen) following the manufacturer's instructions. In brief, recombinant pFastBacHTb–GiPDV 1.1 was transformed into DH10Bac competent cells containing the bacmid and helper plasmid. GiPDV 1.1 was transposed to the bacmid, and insertion of the GiPDV 1.1 caused disruption of lac Z gene, and thus the recombinant bacmid appeared as white colonies on Luria Bertani (LB) plates in the presence of blue-gal, isopropyl-L-thio-β-galactoside, and antibiotics (kanamycin, gentamicin, and tetracycline). Transposition of pFastBacHTb vector without GiPDV 1.1 insert into bacmid was also conducted as a negative control. The recombinant bacmid DNA was purified, and PCR analysis was performed using M13/pUC forward and reverse primers (Invitrogen) to verify the presence and site of insertion of GiPDV 1.1 in the bacmid.

Transfection. For characterization of GiPDV 1.1 expression in insect cells, recombinant bacmid–GiPDV 1.1 was transfected into 12 insect cell lines (Table 1) individually. Each cell line was seeded into eight 35-mm cell culture dishes at 1×10^5 cells in 2 ml of Sf-900 II serum-free-medium (SFM) containing penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were allowed to attach for 2 h at room temperature before transfection. For each transfection, 2.5 μg of recombinant bacmid–GiPDV 1.1 and 6 μl of CELLFECTIN reagent (Invitrogen) were diluted into 100 μl of Sf-900 II SFM without antibiotics individually. The two solutions were mixed and incubated for 45 min at room temperature for the formation of lipid–DNA complexes, followed by addition of 0.8 ml of Sf-900 II SFM. After the cells were washed two times with Sf-900 II SFM without antibiotics, 1 ml of the transfection reagent and recombinant bacmid–GiPDV 1.1 mixtures were added to each dish and allowed to incubate cells for 5 h in a 26° C incubator. After incubation, the mixtures were replaced with 2 ml of Sf-900 II SFM with antibiotics. Transfected cells were incubated in a 26° C incubator for an additional 48, 72, 96, or 120 h.

Two dishes of each insect cell were collected at four time points, 48, 72, 96, and 120 h after transfection. Cells that grew in suspension were transferred from the culture dish to a clean tube using a sterile pipette, whereas tightly attached cells were scraped first and then pipetted out along with culture medium. After removing and saving 100 μl of cell culture medium from each transfection as a master virus stock, cells were harvested by centrifugation for 5 min at $500 \times g$ and immediately subjected to ribonucleic acid (RNA) extraction and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis.

Analysis of protein expression kinetics by RT-PCR. Cell pellets were homogenized in 500 ml of Trizol Reagent (Invitrogen) for total RNA isolation according to the manufacturer's instructions. The RNA samples were resuspended in Diethylpyrocarbonate (DEPC)-treated water in the presence of ribonuclease inhibitor (Invitrogen).

The RNAs extracted from the 12 transfected insect cell lines at each time point after transfection were subjected to RT-PCR analysis using the Access RT-PCR system (Promega). The sequences of forward and reverse primers that were used to amplify a 935-bp product were 5′-ccatattgacgttgctcatc-3′ and 5′-taggtgacaaactcggttgt-3′, respectively. The reaction mixture contained: 1× avian myeloblastosis virus (AMV)/Tfl reaction buffer, 0.2 m*M* each deoxynucleoside triphosphates, 1 μ*M* each forward and reverse primer, 2 m*M* $MgSO_4$, 0.1 unit AMV reverse transcriptase, 0.1 unit T*fl* DNA polymerase, and 500 ng total RNA in a total volume of 50 μl. Amplification was performed using the PTC-100 DNA Engine (MJ Research, Waltham, MA) with the following thermal cycle profile: one cycle of 48° C for 45 min for reverse transcription, one cycle of 95° C for 2 min, 40 cycles of 95° C for 30 s, 55° C

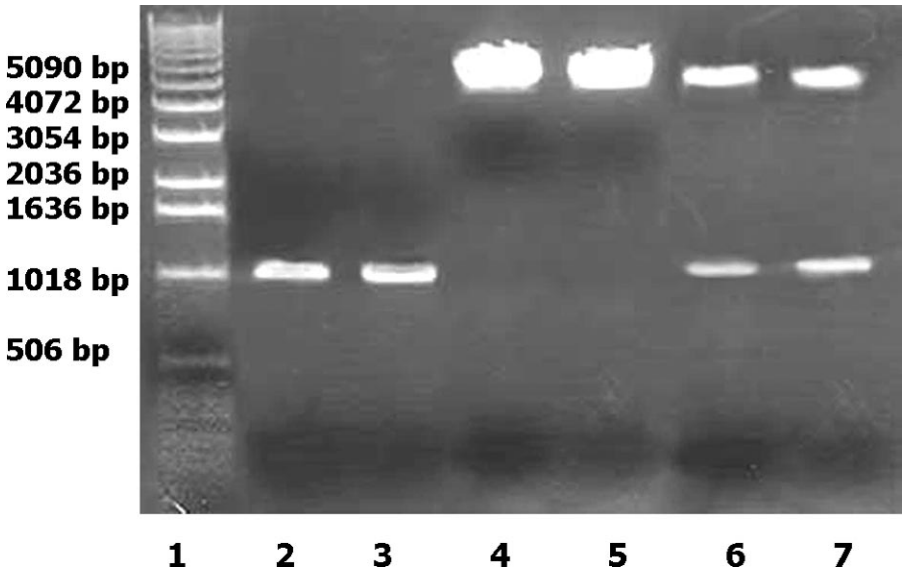

FIG. 1. Identification of GiPDV 1.1 fragment in donor plasmid, pFastBacHTb. Two restriction enzymes, *Nco*I and *Hind*III, were used to cleave GiPDV 1.1 fragment (*lanes* 2, 3) and to linearize the pFastBacHTb plasmid (*lanes* 4, 5) separately. GiPDV 1.1 was cloned at the *Nco*I and *Hind*III sites of pFastBacHTb to form recombinant pFastBacHTb–GiPDV 1.1. When recombinant pFastBacHTb–GiPDV 1.1 was digested with *Nco*I and *Hind*III, it yielded two-sized fragments: a 1002-bp GiPDV 1.1 fragment and a 4.8-bp linear FastBacHTb fragment (*lanes* 6, 7). *Lane 1,* 1-kb deoxyribonucleic acid (DNA) ladder. The positions of kilobase DNA ladder are marked on the *left*.

for 1 min, and 68° C for 2 min, one cycle of 68° C for 7 min. Negative (H_2O) and positive (RNA that was extracted from *G. indiensis* parasitized gypsy moth larvae and proved to be GiPDV 1.1 positive previously) controls were included in each run. The absence of contaminating genomic DNA in the RNA samples was verified by running PCR directly without reverse transcription. The PCR products were analyzed on a 1% agarose gel stained with ethidium bromide and observed under UV light.

Amplification of baculovirus stocks. After maximal expression time points were determined, master virus stocks of transfected cell lines at 96 h after transfection were chosen for amplified recombinant protein production. Hundred microliters of master virus stock from each cell line was used to infect 1×10^5 of the same cells seeded in a 35-mm cell culture dish in 2 ml of Sf-900 II SFM containing antibiotics. Amplification of virus stock for each cell line was duplicated in two dishes. The culture medium was harvested from infected cells after 72 h incubation at a 26° C. The cell culture mediums of the same insect cells from two dishes were combined into one tube. Cells were collected at 500 × *g* centrifugation for 5 min and resuspended in Phosphate-buffered saline and stored at −80° C for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis.

The SDS-PAGE and Western analysis. Individual transfected cell pellets were resuspended in 500 μl of lysis buffer (6 *M* Guanidine HCl; 20 m*M* $NaPO_4$, pH7.8; 500 m*M* NaCl). Cell lysate was passed through an 18-gauge needle three to four times and centrifuged at 3000 × *g* for 20 min to remove cell debris. Supernatant from each was transferred to a fresh tube. Sixty-two microliters of supernatant was removed from each tube for SDS-PAGE and Western blot analysis to examine the expressed protein. The remaining supernatant was stored at −80° C.

Supernatant from each cell line infected with recombinant bacmid–GiPDV 1.1 and supernatant of uninfected control *Spodoptera frugiperda* cells, Sf9 (Invitrogen) were heated at 70° C for 10 min with NuPAGE Reducing Agent and 4× NuPAGE LDS sample buffer (Invitrogen). The samples were loaded onto 4–12% SDS-PAGE minigel and separated by electrophoresis at 200 V constant for 35 min. A precision protein standard (Bio-Rad, Hercules, CA) was included for estimating molecular weight of the protein. The gel was transferred to nitrocellulose membrane using a Transblot-SD Semi-Dry Transfer Cell (Bio-Rad) at 15 V for 25 min. The membrane was blocked with 5% dry milk in Western wash buffer (Tris, pH 7.5, 10 m*M*; NaCl, 100 m*M*; Tween 20, 0.1%) overnight at 4° C. After briefly rinsing the membrane with fresh Western wash buffer, the blot was incubated with 1:200 dilution of primary antibody (Anti-His mouse antibody, Roche, Indianapolis, IN) for 2 h at room temperature. After two washes with Western wash buffer, the membrane was incubated with 1:5000 dilution of secondary antibody (horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G antibody; Pierce, Rockford, IL) for 1 h at room temperature. The membrane was then washed two times in Western wash buffer. Immunoreactive proteins were detected by incubating the membrane in the mixture of SuperSignal West Pico Stable Perocide solution and Luminol–Enhancer solution (1:1 ratio; Pierce) for 4 min at room temperature.

RESULTS AND DISCUSSION

Recombinant plasmid pFastBacHTb–GiPDV 1.1 cleaved with *Nco*I and *Hind*III generated two-sized fragments: a 1002-bp GiPDV 1.1 fragment and an approximately 4.8-kb linearized FastBacHTb fragment (Fig. 1). The DNA sequence analysis of the positive clone GiPDV 1.1 insert indicated that ATG start codon of GiPDV 1.1 gene was in frame. Transformation of recombinant pFastBacHTB–GiPDV 1.1 into DH10Bac competent cells resulted in the construction of the recombinant bacmid–GiPDV 1.1. Electrophoresis showed amplification of recombinant bacmid transposed with pFastBacHTb without GiPDV 1.1 insert and nonrecombinant bacmid yielded a 2430-bp fragment and a 300-bp fragment, respectively, whereas amplification of recombinant bacmid–GiPDV 1.1 yielded a 3432-bp fragment (Fig. 2).

Expression kinetics at four time points after transfection were analyzed by RT-PCR using gene-specific primers (Fig. 3). All RNA samples extracted from transfected cells were subjected to PCR without reverse transcription before RT-PCR analysis. The negative result indicated that RNA samples were free of genomic DNA contamination.

In comparing the wide range of insect cell lines infected with recombinant bacmid–GiPDV 1.1, the gene was expressed earlier in cell line IPLB-PxE2 (derived from *Plutella xylostella*) than in any other cell line tested. During the first 48 h after infection, except for the very faint band detected in the IPLB-PxE2, there was no GiPDV 1.1 expression observed in lysates of other infected insect

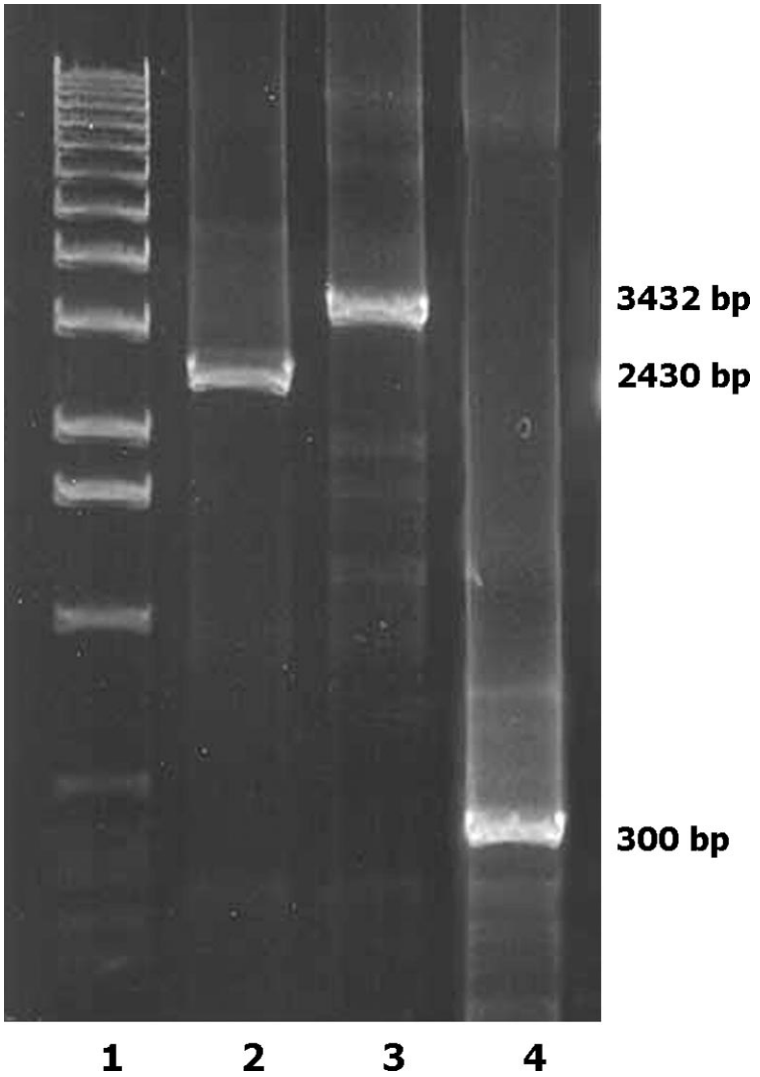

FIG. 2. Polymerase chain reaction identification of recombinant bacmid–GiPDV 1.1. Transformation of recombinant pFastBacHTB–GiPDV 1.1 into DH10Bac competent cells resulted in the construction of the recombinant bacmid–GiPDV 1.1. The PCR analysis was performed to verify the presence of GiPDV 1.1 and correct insertion within the bacmid using M13/pUC forward and reverse primers. *Lane 1,* 1-kb deoxyribonucleic acid ladder. *Lane 2,* recombinant bacmid transposed with donor plasmid, pFastBacHTb (2430 bp). *Lane 3,* recombinant bacmid transposed with pFastBacHTb–GiPDV1.1 (3432 bp). *Lane 4,* blank bacmid (~300 bp). The sizes of fragments are indicated on the *right.*

cell lines. Gene expression was detected in the lysates of all 12 insect cell lines at 72 h after transfection. The maximal level of expression was reached in all the cell lines at 96 h after transfection and thereafter declined. No significant signal was detected, and only a few multiple faint bands were observed in some infected insect cells 120 h after transfection. The expression of GiPDV 1.1 at 72 h after transfection was weaker in two cell lines, UFL-Ag-286 and IPLB-PxE2, than in any other cell lines tested. This may have been because of the nonspecific amplification associated with the GiPDV 1.1 transcript, which could have contributed to reduction product in these two cell lines or possibly an effect of using the Sf-900II medium because both these lines are typically maintained in serum-supplemented media. We chose to use a single medium for all transfections to remove variability in transfection levels or production because of nutrient factors. Admittedly, this could have introduced variability because the specific cell lines were not adjusted to growth in this medium, but our experience suggests all the cell lines used in this study can be maintained on Sf900II for the length of these tests without negative effects. Comparison of the ability of insect cell lines to support expression of GiPDV 1.1 indicated similarity for the cell lines tested at 96 h after transfection. The observation that levels of gene expression between 72 and 96 h after transfection were optimal was consistent with the general pattern observed in other studies, where protein production usually commences after a 3-d infection. This study reinforces the observation that 72–96 h after transfection is the ideal time period for harvest of expressed PDV protein and applies it to a wide range of insect cells.

The expression of GiPDV 1.1 protein in insect cells was confirmed by Western blot analysis, in which GiPDV 1.1 encoded pro-

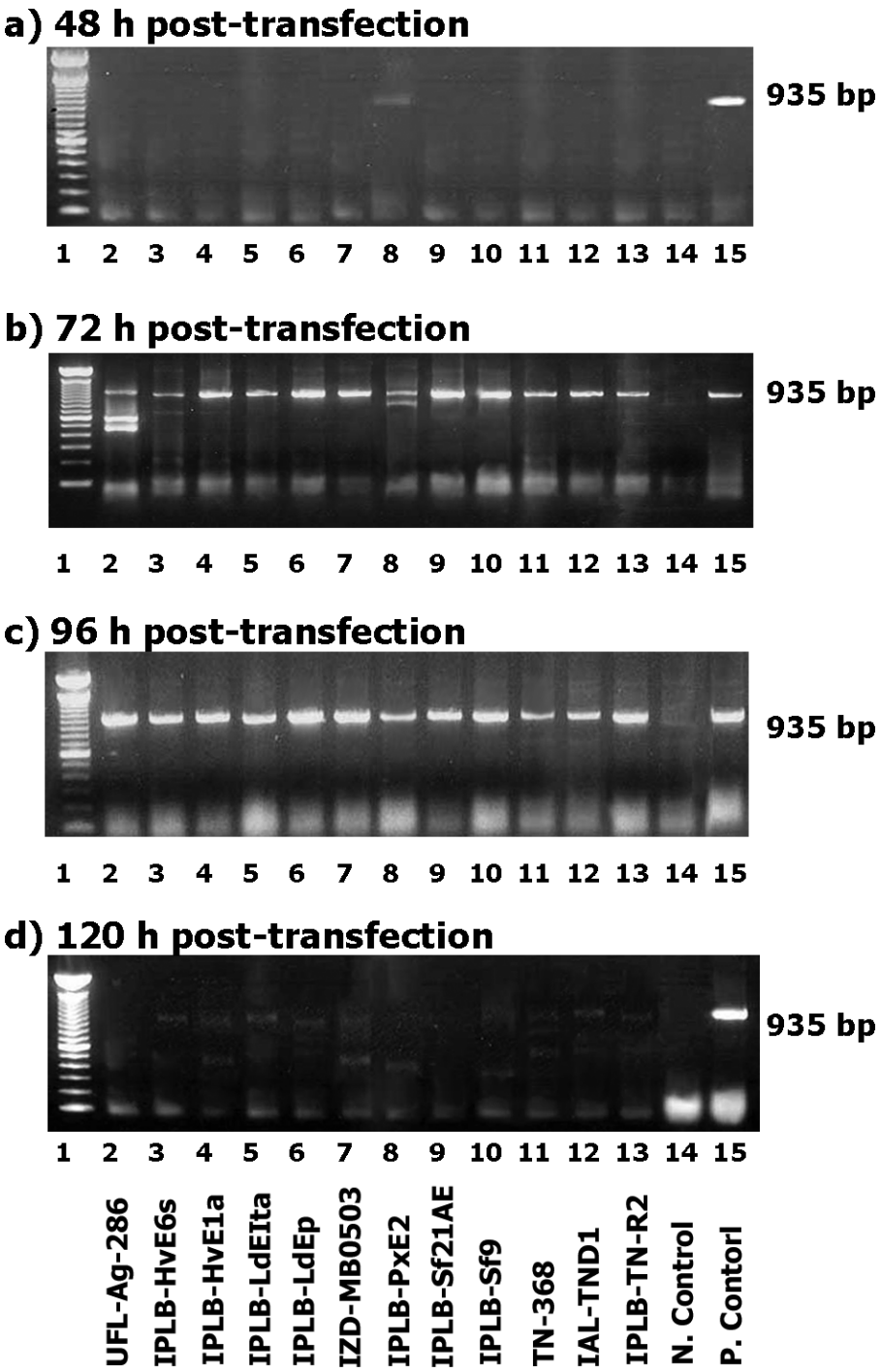

FIG. 3. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of GiPDV 1.1 expression in 12 insect cell lines at four time points after transfection: *a*) 48 h after transfection, *b*) 72 h after transfection, *c*) 96 h after transfection, and *d*) 120 h after transfection. Abbreviation of cell lines are as given in Table 1. Negative (H_2O) and positive (ribonucleic acid that was extracted from *Glyptapanteles indiensis* parasitized gypsy moth larvae and previously proved to be GiPDV 1.1 positive) controls were included in each run of RT-PCR. The sizes of PCR fragments are indicated on the *right*.

tein of the appropriate size was detected in all 12 insect cell lines infected with recombinant bacmid–GiPDV 1.1 but not uninfected Sf9 cells (Fig. 4). A single clear band approximately 40 kDa in size was observed for each infected line. This corresponded well with the calculated molecular weight of GiPDV 1.1 (including the 6X-histidine tag, spacer region, and amino terminus rTEV protease site) of 40 kDa. The very slight differences in molecular mass among the expression products of the range of insect cell lines may have reflected slight differences among posttranslational modifications, such as glycosylation properties of the recombinant protein in each

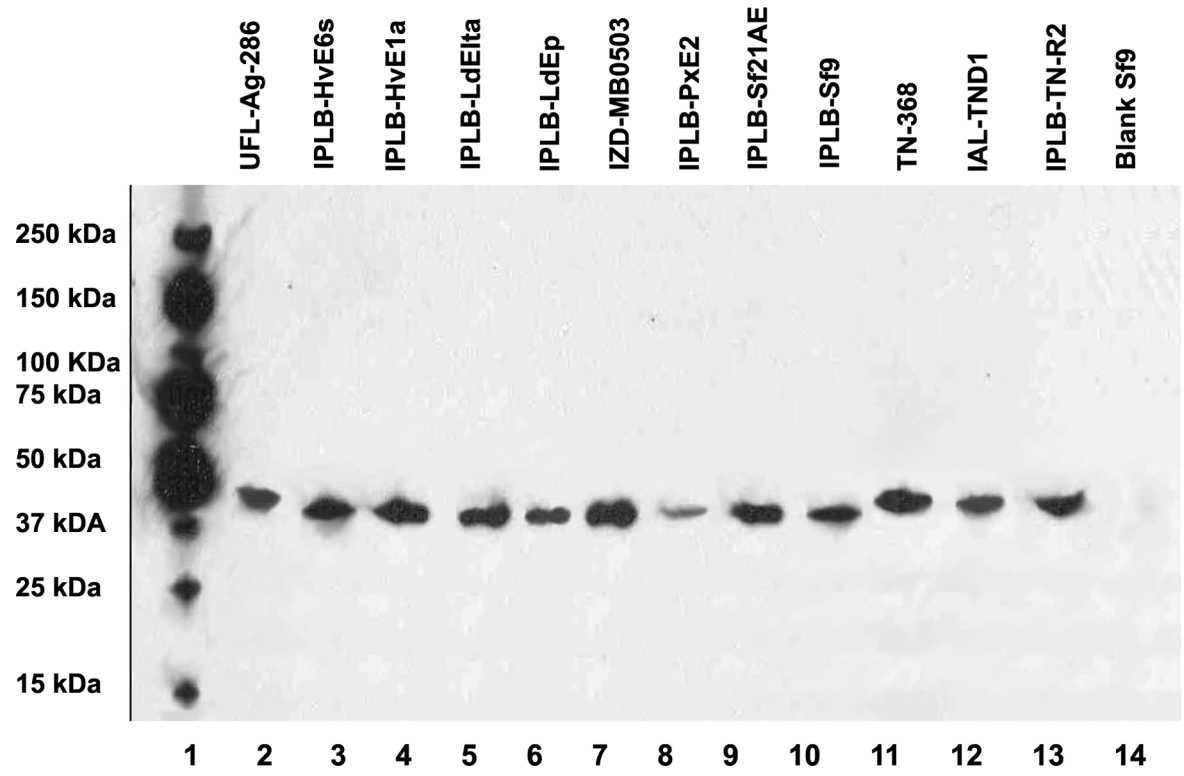

FIG. 4. Western blot analysis of GiPDV 1.1 encoded protein expressed in insect cell lines. *Lane 1,* precision protein standard. The positions of the marker protein are labeled on the *left*. *Lanes 2–13,* cell lysates of 12 insect cell lines infected with recombinant bacmid–GiPDV 1.1. *Lane 14,* cell lysate of blank Sf9 cells. Abbreviation of cell lines are as given in Table 1.

insect cell line. The predicted GiPDV 1.1 gene has six potential *N*-linked glycosylation sites, indicating it may be glycosylated. Previous studies have shown most expressed PDV proteins with *N*-glycosylation sites are glycosylated (Beckage et al., 1987, 1994; Harwood and Beckage, 1994; Harwood et al., 1994; Li and Webb, 1994; Yamanaka et al., 1996; Soldevila et al., 1997; Cui et al., 2000), and minor differences among lines in posttranslational modification would be expected.

The baculovirus expression vector system has been used successfully for production of recombinant PDV proteins (Li and Webb, 1994; Soldevila et al., 1997; Cui et al., 2000). Production of PDV proteins has usually been performed in the two most widely used insect cell lines, Sf9 and Sf21, although a number of other insect cell lines have been established and used for production of various recombinant proteins (Hink et al., 1991; Davis et al., 1993). Our current study included several of the same cell lines used in these previous studies but also included some not previously used with baculovirus expression vectors including IPLB-HvE6s, IPLB-HvE1a, IPLB-LdEP, IPLB-PxE2, and IAL-TND1, although each of the lines was previously shown to be susceptible to the wild type *Autographa californica* nucleopolyhedrovirus that is used in the expression vector system (Lynn, 2003).

Previous studies on transformation of insect cell lines by GiPDV showed many lepidopteran cell lines derived from different tissues permissive to infection (Gundersen-Rindal et al., 1999). However, the ability of these cell lines to support GiPDV gene expression was not examined. Studies by Davis et al., on the production of recombinant protein in various insect cell lines demonstrated cells derived from embryonic tissues of cabbage looper, *Trichoplusia ni* (High 5™ cells), were often a better choice for production of recombinant protein than Sf9 cells (Davis et al., 1993). This was not the case for production of GiPDV 1.1, where most insect cell lines supported production of similar quantities of recombinant protein. Thus, a wide range of insect cells were suitable for Bac-to-Bac baculovirus–based expression of recombinant PDV protein, and many lepidopteran cell lines could serve as alternative sources to the commonly used *S. frugiperda* Sf9 and Sf21 cells.

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